

**Remarks/Argument**

By the present amendment, claims 1 and 22-23 have been amended, and claim 38 has been added. Support for amended claims 1 and 22-23, as well as new claim 38 can be found at at least p. 11, lines 3-25, and p. 13, line 28 to p. 14, line 1 of the present application.

Below is a discussion of the February 23, 2010 telephone conversation with Examiner Sgagias, the 35 U.S.C. §112, first paragraph, rejection of claims 1-2, 5, 7, 21-23 and 37, and the patentability of new claim 38.

**1. February 23, 2010 telephone conversation.**

Applicants wish to memorialize the February 23, 2010 telephone conversation with Examiner Sgagias in which Applicants' proposed claim amendments were discussed. Examiner Sgagias informed Applicants that she could not say whether the proposed claim amendments would overcome the outstanding 35 U.S.C. §112, first paragraph, rejection without further searching. Examiner Sgagias suggested that Applicants file an After File Amendment including the proposed claim amendments. Examiner Sgagias agreed that she would review the After Final Amendment and then inform Applicants as to whether she would enter the After Final Amendment or require Applicants to file an RCE.

**2. 35 U.S.C. §112, first paragraph, rejection of claims 1-2, 5, 7, 21-23, and 37**

Claims 1-2, 5, 7, 21-23, and 37 were rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Office Action argues the claims embrace an enormous number of integrin-receptor binding

fragments constituting a claimed genus. Thus, the Office Action argues, the claims embrace a claimed genus that encompasses integrin-receptor binding fragments yet to be discovered.

By the present amendment, claims 1 and 23 have been amended to recite "vitronectin (VN) or an  $\alpha_v$  integrin-receptor binding fragment thereof that does not comprise a heparin binding domain (HBD)." Applicants respectfully submit that the amendments to claims 1 and 23 satisfy the requirements of 35 U.S.C. §112, first paragraph, because the present specification sufficiently describes the structure or functional nature of VN or an  $\alpha_v$  integrin-receptor binding fragment thereof that does not comprise a HBD. For example, the present application discloses that the VN fragments may be characterized as: (i) having at least an  $\alpha_v$  integrin-receptor binding region; and (ii) lacking a HBD (see p. 11, lines 3-25). With respect to the  $\alpha_v$  integrin-receptor binding (or RGD) region of VN, this has been studied in numerous papers published prior to the priority date of the present invention. In fact, this domain has been mapped as far as the specific residues of VN that activate signaling pathways (see, e.g., Seger *et al.*, *J Biol Chem.*, 273(38):24805-24813, 1998; attached hereto). Additionally, the HBD of VN has been previously identified as the C-terminal region of mature VN (i.e., amino acid residues 347-459).

The subject matter of amended claims 1 and 23 is also fully supported by the disclosure of International Application No. PCT/AU2004/000117 ("the '117 application"), which is incorporated by reference into the present application (see p. 11, line 9). For example, the '117 application discloses a number of different integrin-receptor binding VN fragments (see, e.g., p. 13, line 17 to p. 15, line 20).

Additionally, the '117 application discloses VN fragments that do not comprise the HBD of mature VN (*i.e.*, amino acid residues 347-459) (see p. 35, lines 19-23 and Fig. 8), as well as specific examples of biologically active integrin receptor-binding VN fragments that lack the HBD (see p. 15, lines 7-8 and Fig. 14).

Accordingly, Applicants respectively submit that the subject matter of amended claims 1 and 23 is described with sufficient particularity such that one of ordinary skill in the art would recognize that Applicants were in possession of the claimed the subject matter at the time of invention, and request that the 35 U.S.C. §112, first paragraph, rejection of claims 1 and 23 be withdrawn. Additionally, Applicants respectively request that the 35 U.S.C. §112, first paragraph, rejection of claims 2, 5, 7 and 21-22, which depend directly from claim 1, be withdrawn.

**3. Patentability of new claim 38.**

By the present amendment, Applicants have added claim 38. New claim 38 depends directly from claim 1 and recites the further feature that the  $\alpha_v$  integrin-receptor binding fragment comprises amino acid residues 1-52 of mature VN.

Applicants respectively submit that new claim 38 satisfies the requirements of 35 U.S.C. §112, first paragraph, because one skilled in the art at the time of the present invention would have understood that the Applicants were in possession of an  $\alpha_v$  integrin-receptor binding fragment comprising amino acid residues 1-52 of mature VN. Support for new claim 38 can be found at at least p. 11, lines 15-25, and p. 13, line 28 to p. 14, line 1 of the present application. Additionally, one skilled in the art would appreciate that the claims recite VN fragments that are: (i) capable

of binding to an  $\alpha_v$  integrin-receptor (*i.e.*, include at least an RGD) and (ii) lack the HBD. From this, the skilled artisan would understand that these features inevitably lead to a VN fragment that comprises amino acid residues 1-52 of mature VN.

Accordingly, Applicants respectively submit that new claim 38 satisfies the requirements of 35 U.S.C. §112, first paragraph, and allowance of new claim 38 is requested.

Please charge any deficiency or credit any overpayment in the fees for this matter to our Deposit Account No. 20-0090.

Respectfully submitted,

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# Phosphorylation of Vitronectin by Casein Kinase II

IDENTIFICATION OF THE SITES AND THEIR PROMOTION OF CELL ADHESION AND SPREADING\*

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The cell adhesion protein vitronectin (Vn) was previously shown to be the major target in human blood for an extracellular protein kinase A, which is released from platelets upon their physiological stimulation with thrombin and also prevails as an ectoenzyme in several other types of blood cells. Because plasma Vn was shown to have only one protein kinase A phosphorylation site (Ser<sup>378</sup>) but to contain ~3 mol of covalently bound phosphate, and because human serum and blood cells were shown to contain also a casein kinase II (CKII) on their surface, we studied the phosphorylation of Vn by CKII attempting to find out whether such phosphorylation modulates Vn function, an acid test for its having a physiological relevance. Here we show (i) that the CKII phosphorylation of Vn has a  $K_m$  of 0.5–2  $\mu$ M (lower than the Vn concentration in blood, 3–6  $\mu$ M), (ii) that it is targeted to Thr<sup>50</sup> and Thr<sup>57</sup>, which are vicinal to the RGD site of Vn, and (iii) that the phosphorylation of Thr<sup>57</sup> facilitates the phosphorylation of Thr<sup>50</sup>. The maximal stoichiometry of the CKII phosphorylation of plasma Vn was found to be low, which, in principle, could be due to its partial prephosphorylation *in vivo*. However, for the detection of a functional modulation, we needed a comparison between a fully phosphorylated Vn (at Thr<sup>57</sup> and Thr<sup>50</sup>) and a nonphosphorylated Vn. Therefore, we expressed Vn in a baculovirus system and show (i) that the CKII phosphorylation of wt-Vn enhances the adhesion of bovine aorta endothelial cells; (ii) that the double mutant T50E/T57E (in which the neutral Thr residues are replaced by the negatively charged Glu residues considered analogs of Thr-P) has a significantly enhanced capacity to promote cell adhesion and to accelerate cell spreading when compared with either wild-type Vn or to the neutral T50A/T57A mutant; and (iii) that, at least in the case of bovine aorta endothelial cells, the T50E/T57E mutant exhibits an enhanced adhesion, which seems to be due to an increased affinity toward the  $\alpha_1\beta_3$  Vn receptors.

Originally discovered as a "serum spreading factor" (1), vitronectin (Vn)<sup>1</sup> is now considered an important adhesive gly-

coprotein in the extracellular matrix of various cells and in circulating blood (2–4). It promotes cell attachment, spreading, and migration (5–8) through an RGD sequence that is known to be recognized by the integrins (9, 10). In addition, Vn was shown to participate in the regulation of the complement function (11–15) and in the control of plasminogen activation, by stabilizing the inhibitory conformation of the inhibitor-1 of plasminogen activators, leading to an arrest of the production of plasmin from plasminogen (16–22).

We have previously shown that Vn is the major protein substrate in serum for protein kinase A (PKA), which is also found as an ectoenzyme in several types of blood cells (23, 24).<sup>2</sup> PKA was shown to be released into the blood fluid from platelets upon their activation with thrombin (24, 25). The distinct selectivity of this Vn phosphorylation by PKA and the finding that this kinase is released from the platelets in response to a key physiological (thrombin) stimulus in hemostasis suggested to us that this kinase-driven covalent modification may have a physiological regulatory importance. Support for this notion came from reconstitution experiments using Vn isolated from human plasma and a pure catalytic subunit of PKA. These experiments revealed that under the conditions existing at the locus of a hemostatic event, the phosphorylation of Vn by PKA may well take place *in vivo* (26–28). Moreover, we found that following this PKA phosphorylation, there is a conformational change in Vn (which was demonstrated by circular dichroism) that results in a significant reduction in the binding of Vn to the inhibitor-1 of plasminogen activators (29) and, consequently, in the modulation of an important physiological function of Vn.

The specificity (Ser<sup>378</sup> only) and stoichiometry of the PKA phosphorylation of plasma Vn as purified from human blood (24, 26, 28, 30, 31) suggested to us that *in vivo*, Vn is most likely phosphorylated also by other protein kinases (besides PKA, because plasma Vn was found to contain ~3 mol of covalently bound phosphate per mol of Vn (30, 31)). Indeed, we have recently shown that *in vitro* Vn is also a substrate for PKC, which phosphorylates it at Ser<sup>362</sup> (again with a stoichiometry of ~0.7 mol/mol), and subsequently attenuates the cleavage of Vn by plasmin (32). However, if we take together these PKA and PKC phosphorylations, they are not sufficient to account for the stoichiometry of ~3 mol of phosphate per mol of Vn, which was determined prior to the *in vitro* phosphorylation of the vacant phosphorylation sites of Vn. Therefore, we assumed that *in vivo*, there might still be an additional kinase (and maybe more) that might phosphorylate Vn. In fact, this possibility seemed plausible on the basis of our earlier findings that

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<sup>2</sup> The abbreviations used are: Vn, vitronectin; CKII, casein kinase II;

PKA, protein kinase A; wt, wild type; r-Vn, recombinant Vn; BAE, bovine aorta endothelial cell; AMP-PCP, adenosine 5'- $\beta$ -methylentriphosphate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter.

<sup>2</sup> A. Belletti, M. Bravo, and S. Shaltiel, unpublished results.

(a) upon fractionation of human serum on an anion-exchange column, we identified three peaks of protein kinase activity one of which phosphorylates phosvitin (33), and (b) that the phosphorylation of Vn by platelet ectokinases was not completely inhibited by the PKA and PKC inhibitors PKI and calphostin C.<sup>3</sup> A plausible candidate for this purpose seemed to be CKII, because a protein kinase activity with a CKII-like specificity was found in serum (33) and, as an ectoenzyme, on epithelial cells (34, 35), neutrophils (36, 37), endothelial cells (38, 39), and platelets (40, 41).<sup>3</sup>

Here, we report the biochemical characterization of the CKII phosphorylation of Vn, focusing on its possible physiological significance. Plasma Vn is shown to be a substrate for CKII and to phosphorylate Thr<sup>50</sup> and Thr<sup>57</sup> (i.e. amino acid residues vicinal to the cell-adhesion RGD site of Vn (positions 45–47)). The functional consequences (and thus the possible physiological relevance) of this CKII phosphorylation is demonstrated by comparing wt-Vn to the CKII phosphorylated wt-Vn, as well as the two double mutants: T50E/T57E (a mutant representing an analog of a fully phosphorylated Vn at Thr<sup>50</sup> and Thr<sup>57</sup>) and T50A/T57A (a mutant representing an analog of a nonphosphorylated Vn at these two positions). The adhesion of bovine aorta endothelial cells (BAECs) onto the CKII phosphorylated wt-Vn or onto its T50E/T57E mutant is shown to be enhanced (via an  $\alpha_1\beta_2$  integrin) in comparison to their adhesion onto the nonphosphorylated wt-Vn, as well as onto its T50A/T57A mutant.

#### MATERIALS AND METHODS

**Chemicals, Materials, Antibodies, and Enzymes.**—The following were purchased from the commercial sources: phosphoserine, phosphodervative, phosphotyrosine, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) from Sigma; [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) and [ $^{35}$ S]methionine from Amersham Pharmacia Biotech; nitrocellulose membranes from Schleicher & Schuell; polyvinylidene difluoride membranes from Millipore; thin layer cellulose plates (20  $\times$  20 cm) from Merck; restriction enzymes from Boehringer Mannheim or Life Technologies, Inc.; *Tag* DNA polymerase from Promega; the baculovirus linear DNA from PhaMingen; recombinant human CKII (expressed in *E. coli*) from Boehringer Mannheim or Calbiochem; poly-L-lysine (average molecular mass 70–150 kDa); and  $\alpha$ -thrombin from Sigma. Monoclonal antibodies (P1P6) directed against the integrin receptor  $\alpha_1\beta_2$  and LM609 directed against the integrin receptor  $\alpha_5\beta_1$  were obtained from Chemicon. Goat anti-mouse IgG fluorescein isothiocyanate-conjugated antibodies were purchased from Sigma. All other materials were the best commercially available grade.

**Other Proteins and Enzymes.**—Vn was purified from freshly frozen human plasma as described previously (12), with the modifications used routinely in our laboratory (25, 27). The catalytic subunit of PKA was purified according to the method described by Beavo *et al.* (42).

**Thrombin Cleavage of Vn** (28, 43)—The reaction mixture (50  $\mu$ l) contained the following components at the final concentrations given in parentheses: CKII phosphorylated Vn (50  $\mu$ g/ml), thrombin (30 IU/ml), and Hepes buffer (30 mM, pH 7.5). The reaction was allowed to proceed for 30 min at 37 °C and was arrested by the adding 12  $\mu$ l of 5 $\times$  Laemmli's sample buffer, boiling for 3 min, and then subjecting it to SDS-PAGE.

**In Vitro Phosphorylation of Vn by CKII.**—The reaction mixture (50  $\mu$ l) contained the following components at the indicated final concentrations: Vn (50–200  $\mu$ g/ml), CKII (2  $\mu$ g/ml), glycerol (5%), NaCl (50 mM),  $\beta$ -mercaptoethanol (5 mM), magnesium acetate or magnesium chloride (10 mM), [ $\gamma$ -<sup>32</sup>P]ATP (10  $\mu$ M, 6–30 Ci/mmol), and Hepes buffer (30 mM, pH 7.5). The reaction was allowed to proceed at 22 °C for 30 min (or the time period indicated in the figure) and was arrested by adding 12  $\mu$ l of 5 $\times$  Laemmli's sample buffer (or 18  $\mu$ l of 4 $\times$ ) and boiling for 3–5 min.

**Increasing the Stoichiometry of the CKII Phosphorylation of wt-Vn by Activation of the Kinase.**—Samples of wt-Vn or T50A/T57A-Vn were phosphorylated *in vitro* by CKII in the following reaction mixture: CKII (2  $\mu$ g/ml), glycerol (5%), NaCl (50 mM),  $\beta$ -mercaptoethanol (5 mM), magnesium chloride (10 mM), [ $\gamma$ -<sup>32</sup>P]ATP (10  $\mu$ M, 6–30 Ci/mmol), Hepes buffer (30 mM, pH 7.5), in the absence or presence of 15  $\mu$ g/ml poly-L-

lysine. The reaction was allowed to proceed at 22 °C for 30 min, arrested by boiling for 5 min in Laemmli's sample buffer, then loaded on SDS-polyacrylamide gels (10% acrylamide). The gel was dried and exposed to autoradiography, and the radioactive phosphorylated Vn bands were excised from the dried gel and counted in a scintillation counter. The stoichiometry of phosphorylation was calculated on the basis of the specific radioactivity of [ $\gamma$ -<sup>32</sup>P]ATP.

**Preparation of Phosphorylated wt-Vn and Nonphosphorylated wt-Vn for Cell Adhesion Assays.**—The reaction mixture was prepared as described above except for the fact that [ $\gamma$ -<sup>32</sup>P]ATP was replaced by nonradioactive ATP. For the preparation of nonphosphorylated wt-Vn, ATP was substituted by AMP-PCP. All other constituents were the same. The reaction was allowed to proceed at 22 °C for 30 min; thereafter, the reaction mixture was serially diluted and applied onto 48-well plates for the cell adhesion assay.

**Acid Cleavage of Vn—Vn was phosphorylated by CKII as described above and subjected to SDS-PAGE (10% acrylamide). The gel was electroblotted onto nitrocellulose membrane, and the membrane was then washed with distilled water and exposed to autoradiography. The Vn bands were localized in the membrane by superimposing the membrane onto the autoradiogram, excised, and transferred into a microtube containing 100  $\mu$ l of 70% formic acid. After incubation for 12 h at 37 °C, the extract was diluted with double distilled water 7–10 fold, concentrated in a Speedvac, dissolved in Laemmli's sample buffer, boiled (3 min), and subjected to SDS-PAGE.**

**Phosphoaminoacid Analysis of Phosphorylated Vn—Phosphoaminoacid analysis was carried out as described previously (44).**

**Purification and Sequencing of a Tryptic Peptide from Vn Harboring the CKII Phosphorylation Site.**—A sample of Vn (2 nmol), was heated to 56 °C for 15 min to allow maximal CKII phosphorylation (cf. Fig. 1) and then chilled on ice. Phosphorylation by CKII (0.5  $\mu$ g) was allowed to proceed for 60 min at 22 °C in a reaction mixture (200  $\mu$ l) containing magnesium acetate (10 mM), [ $\gamma$ -<sup>32</sup>P]ATP (10  $\mu$ M, 6 Ci/mmol), and Hepes (50 mM, pH 7.5). The reaction was arrested by an addition of a 50-fold excess of nonradioactive ATP. Then, Vn was reduced (15 min with 10 mM dithiothreitol, pH 7.5, at 56 °C), alkylated with 30 mM iodacetamide (for 1 h in the dark), and precipitated by ammonium sulfate (70%) to separate the protein from the excess radioactive ATP; it was then extensively dialyzed overnight at 4 °C against 50 mM Hepes, pH 7.4, and digested with trypsin (sequencing grade, 5  $\mu$ g) at 36 °C for 6 h. The material was diluted 1:10 with double distilled water containing 0.1% trifluoroacetic acid, applied to a C<sub>8</sub> reverse phase-high pressure liquid chromatography column, and eluted by a linear gradient of acetonitrile (0–80%) for 120 min. The flow rate was 0.33 ml/min. The absorbance peaks (214 nm) were collected manually and counted on a scintillation counter. The peaks that contained the highest amount of radioactivity were selected for amino acid sequence analysis.

**Tissue Cultures.**—BAECs were grown in tissue culture dishes in DMEM (low glucose; 1% gliter) supplemented with 10% (v/v) calf serum (Life Technologies, Inc.). NIH-3T3 cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated calf serum (HyClone). The cells growing was carried out in an incubator (37 °C) with a atmosphere containing 5% CO<sub>2</sub>. The SF9 and High-5 insect cells were maintained in Grace's insect medium (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum. For the expression of recombinant Vns, a serum-free medium (SF-900 II, Life Technologies, Inc.) was used. All media were supplemented with 50  $\mu$ g/ml Gentamicin and 12.5  $\mu$ g/ml Fungizone (Life Technologies, Inc.).

**Adorption of Vn onto the Plastic Surface of Microtiter Plates for Enzyme-linked Immunosorbent Assay.**—Vn solutions serially diluted in PBS were added to 96-well microtiter plates for 2 h at 22 °C, and the residual nonspecific adsorption sites were blocked with 0.3% bovine serum albumin in PBS for 1 h. Bound Vn was determined with specific rabbit anti-human Vn polyclonal antibodies and goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1 h at 22 °C). After extensive washings with PBS containing 0.01% Tween 20, the chromogenic substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was added. The reaction was monitored in an enzyme-linked immunosorbent assay reader at 620 nm.

**Cell Adhesion Assay.**—Serial dilutions of Vns were added to 24-well plates (250  $\mu$ l) for 1.5 h at 22 °C to allow coating of the plates. Thereafter, the solutions were aspirated, and 0.5 ml of DMEM containing 1 mg/ml hemoglobin was added for 30 min at 37 °C. Confluent BAECs or NIH-3T3 cells plated on 10-cm plates were labeled with 30  $\mu$ Ci [<sup>35</sup>S]methionine for 3–4 h at 37 °C. The cells were then trypsinized and collected into DMEM containing 0.2 mg/ml soybean trypsin inhibitor to arrest trypsin action. The cells were then centrifuged (5 min at 1200  $\times$  g) and resuspended in DMEM containing 1 mg/ml hemoglobin adjusting

the cell concentration to  $10^6$  cells/ml. Cell suspensions (250  $\mu$ l) were added to each coated well for 30 min at 37 °C. The cells were washed three times with 0.5 ml of PBS, and the adhered cells were treated with 0.5 ml of 1% Triton X-100 in PBS for 5 min. Samples of 0.4 ml were transferred into scintillation vials for counting. The quantitation of cell adhesion is reported as the residual radioactivity (a mean of triplicates in cpm) of the cells to be tested, after their extensive washing (three times with 0.5 ml of PBS). This comparison was convenient and valid, because each assay was carried out with an identical volume of cell suspension and an identical number of cells (~700,000 cpm/250,000 cells). Cell adhesion assays of the nonphosphorylated wt-Vn and the CKII phosphorylated wt-Vn were performed in 48-well plates. All of the components and treatments of the assay were scaled down accordingly.

**Inhibition of BAEC Adhesion by Function-inhibiting Monoclonal Antibodies.**—The monoclonal antibodies used were as follows: P1F6, directed against the integrin receptor  $\alpha_5\beta_1$ ; LM609, directed against the integrin receptor  $\alpha_5\beta_1$  and HA, directed against hemagglutinin as control. Plates (24 wells) were coated with a 5  $\mu$ g/ml concentration of the Vn to be assayed (250  $\mu$ l) for 1.5 h 22 °C, and then the nonspecific adsorption sites were blocked with 0.5 ml of DMEM containing 1 mg/ml hemoglobin (30 min at 37 °C). The cells were treated as described above (under "Cell Adhesion Assay") and resuspended in DMEM containing 1 mg/ml hemoglobin to yield a concentration of  $10^6$  cells/ml. Before starting the cell adhesion assay, the cells were preincubated with different amounts of monoclonal antibodies (with gentle shaking) as indicated in the legend to Fig. 8 (30 min at 22 °C). Thereafter, the cells were washed once with 10 ml of DMEM containing 1 mg/ml hemoglobin and resuspended to yield a concentration of  $10^6$  cells/ml. An aliquot of this cell suspension (250  $\mu$ l) was added to the Vn-coated wells, and the adhesion assay was allowed to proceed as described above.

**FACS Analysis of  $\alpha_5\beta_1$  and  $\alpha_5\beta_1$  Expression in BAECs—Confluent BAECs were grown on 10-cm plates and then trypsinized and suspended in DMEM containing 0.2 mg/ml soybean trypsin inhibitor to arrest trypsin action. The cells were then centrifuged (5 min at 1200  $\times$  g) and resuspended in DMEM containing 1 mg/ml hemoglobin to a concentration of  $5 \times 10^6$  in 100  $\mu$ l. The cells were incubated with anti- $\alpha_5\beta_1$  or with anti- $\alpha_5\beta_1$  monoclonal antibodies (final concentration, 4  $\mu$ g/100  $\mu$ l) for 1 h on ice with occasional agitation. They were then washed three times with 1 ml of a PBS buffer containing 1% bovine serum albumin and 0.02% sodium azide using a cooled microcentrifuge (4 °C). After the last wash, the cells were resuspended in 100  $\mu$ l of the above-mentioned buffer, supplemented with goat anti-mouse IgG that was conjugated with fluorescein isothiocyanate (final concentration, 5  $\mu$ g/100  $\mu$ l). The cells were allowed to bind the antibodies for 1 h (on ice) with occasional agitation and then washed as above and resuspended in 0.5 ml of PBS buffer (described above) for FACS analysis in a Becton Dickinson FACScan (530 filter). For each antibody, 5000 cells were analyzed. Control cells were incubated with the secondary antibody only.**

**Cell Spreading Assays—**Samples (20  $\mu$ g) of wild-type or mutant Vn were diluted in PBS to a final volume of 2 ml and added to 6-well plates (Nunc) for 2 h at 22 °C. After coating, the solutions were aspirated, and 0.5 ml of DMEM containing 1 mg/ml hemoglobin was added for 30 min at 37 °C to block the residual nonspecific adsorption sites. BAECs or NIH-3T3 cells (70% confluent) plated on a 10-cm plate were serum-starved for 16 h; thereafter, the cells were trypsinized and brought as described above to a concentration of  $10^6$  cells/ml. A sample (4 ml) of the cell suspension was added to each coated well. After 8 min in an incubator, unattached cells were gently aspirated, and attached cells were supplemented with 4 ml of warmed DMEM (37 °C) containing 1 mg/ml hemoglobin. Spreading of cells was monitored on Zeiss Axiovert 35 microscope containing a heating unit plate TRZ 3700, calibrated at 37 °C. Pictures were taken at the times indicated in Fig. 7.

**Preparation of Recombinant Wild-type Vn for Expression in Insect Cells—**Human Vn cDNA was generously provided by Dr. Erkki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) (45). This clone includes no 5'-untranslated DNA and is missing the first three amino acids; therefore, the transcription initiation sequence (46) and the first three amino acids (Met-Ala-Pro) were added to the clone by PCR. The sense oligonucleotide sequence used is 5'-GGGAGCTC-GCCACCATGGC-CTTCTGAGACCCCTCTCTCATCA-3'.

The antisense oligonucleotide is located downstream the *SacI* site at nucleotide 445. The PCR product was digested with *SacI* and ligated to *SacI*-digested pRSET-wt-Vn. The construct obtained by PCR amplification was sequenced past the *SacI* sites in order to confirm a correct amplification before further subcloning into the PVL1393 transfer vector (PharMingen).

**Mutations of the CKII Phosphorylation Sites in Vn—**All mutants

were obtained by PCR amplification and insertion into an *ACCI* site that was especially designed by a silent mutation near Thr<sup>57</sup> as follows: The two amino acids following Thr<sup>57</sup> are Val<sup>58</sup> and Tyr<sup>59</sup>, which are encoded by GTCTAT. This sequence was changed to GTCTAC, which still encodes for Val and Tyr but also creates an *ACCI* site. The fragments were designed to contain also the mutated threonines using the oligonucleotide sequence given below. The sense oligonucleotide used to prime all mutants was 5'-TAAGAATCCGAGCTGCCAAC-3'. The antisense oligonucleotides that were used to generate each one of the mutations are as follows: for the T50A mutant, 5'-CTCTGACGCCGT-ATCTACATCTCCGGCATAGCGAACACATC-3'; for T50E mutant, 5'-CTCGTAGACCTCTGTACTCATCTCCGGCATAGCGAACACATC-3'; for the T50/A57T double mutant, 5'-CTCGTAGACCCGGCTACTCATCTCCGGCATAGCGAACACATC-3'; and for the T50/E57T double mutant, 5'-GTCGTAGACCTCTGTACTCATCTCCGGCATAGCGAACACATC-3'.

All PCR-amplified fragments were ligated into the *Bam* HI-*ACCI* site of Vn cDNA-pRSET. The construction of the mutations deleted a fragment of 588 base pairs, which was later added to all mutants by PCR amplification of the missing fragment. The pair of oligonucleotides that were used for that purpose were the sense oligonucleotide 5'-TACAG-GTCTACGACCATGCG-3' and the antisense oligonucleotide 5'-CCT-GACTGCGCTGCTGCGA-3'. The PCR-amplified fragment digested with *ACCI* and ligated into the *ACCI*-digested Vn mutant pRSET, which was missing this fragment. All amplified PCR products were sequenced after subcloning in order to ascertain correct amplifications, and they were then ligated into the PVL1393 transfer vector (PharMingen).

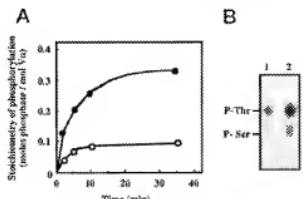
**Forced Recombination into the Viral DNA and Plaque Purification—**Each transfer vector containing Vn cDNA was cotransfected along with linear viral DNA into SF-9 cells as described by PharMingen. Isolated plaques (purified by agarose overlays) as described in Ref. 47 were analyzed and amplified for further use.

**Expression of Vn in High 5 Insect Cells—**High titer virus stocks obtained from purified plaques were used to infect High 5 insect cells. Infected cells were grown in serum-free medium (SF900 II from Life Technologies, Inc.) in order to eliminate contamination of the recombinant Vn with bovine Vn from fetal bovine serum. The expressed Vns, which were secreted into the medium by their own signal, were collected 72 h after the infection.

## RESULTS

**Stoichiometry of the CKII Phosphorylation of Vn—**When Vn (purified from human plasma) was subjected to phosphorylation by CKII in the presence of [ $\gamma$ -<sup>32</sup>P]ATP, an incorporation of <sup>32</sup>P occurred with a stoichiometry of ~0.1 mol of phosphate per mol of Vn (Fig. 1A). If Vn was heat-treated (15 min at 56 °C) prior to the phosphorylation (a treatment that opens up the Vn molecule and favors its multimerization (48)), the stoichiometry of phosphorylation was increased but reached a plateau at ~0.35 mol/mol (Fig. 1A). As seen in Fig. 1B, under both conditions, the phosphorylation was targeted mainly on Thr residues, suggesting that the increased stoichiometry following heat treatment may be due to an increased exposure of the target Thr residues involved. It should be noted that although there is an apparent increase in phosphoserine content in Fig. 1B, the ratio between Thr and Ser phosphorylation before and after heat treatment is essentially the same (<9%, as judged by densitometric scanning). On the other hand, the fact that even after heat treatment, CKII phosphorylated the plasma-purified Vn only up to ~0.35 mol/mol indicated that some of the Vn molecules in plasma may already be prephosphorylated at these Thr residues, and they probably constitute part of the phosphate content of plasma Vn (~3 mol/mol) determined by Tollesfson and co-workers (30). Interestingly, the prephosphorylated Vn appears to be a better substrate for CKII (Fig. 2A), as indicated by the kinetic analysis of the CKII phosphorylation of Vn (Fig. 2B), which showed that the heat treatment results in a 4-fold decrease of the  $K_m$  (from 2 to 0.5  $\mu$ M; Fig. 2B).

**Localization of the CKII Phosphorylation Sites in Vn—**Unlike PKA, which in the absence of heparin, preferentially phosphorylates the one-chain form of Vn (Fig. 3, A and B, lane 1), CKII phosphorylates both the one-chain and the two-chain

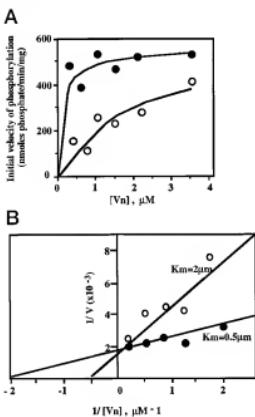


**Fig. 1. The CKII phosphorylation of Vn is targeted to Thr residues.** *A*, time course of CKII phosphorylation of Vn in its native and its heat-treated (open) conformation. Samples (5  $\mu$ g each) of native Vn (open circles) or a heat-treated Vn (15 min at 56 °C) (closed circles) were phosphorylated by CKII in the presence of [ $\gamma$ - $^{32}$ P]ATP (see under "Materials and Methods"). At the times indicated, aliquots (40  $\mu$ l) were removed from the reaction mixture into tubes containing 10  $\mu$ l of 5 $\times$  Laemmli's sample buffer and were boiled for 3 min. The proteins were subjected to SDS-PAGE (10% of acrylamide). The gel was then dried and subjected to autoradiography. The phosphorylated bands of Vn (both the Vn<sub>57</sub> and the Vn<sub>57+65</sub> forms together) were excised from the dried gel and counted in a scintillation counter. The stoichiometry of the phosphorylation was calculated on the basis of the specific radioactivity of the [ $\gamma$ - $^{32}$ P]ATP used and the amount (in mol) of Vn applied in each lane. *B*, phosphoamino acid analysis of the CKII phosphorylated Vn in its native and its heat-treated conformations. Samples (10  $\mu$ g each) of native Vn (lane 1) or heat-treated Vn (lane 2) were phosphorylated by CKII in the presence of [ $\gamma$ - $^{32}$ P]ATP and subjected to hydrolysis and phosphoamino acid analysis as described under "Materials and Methods." The plate was stained with ninhydrin for visualization of the marker phosphoamino acids and then exposed to x-ray film for autoradiography.

forms of Vn (Vn<sub>57</sub>, and Vn<sub>57+65</sub> respectively) (Fig. 3A, lane 2), indicating that the CKII phosphorylation site(s) is located between residues Asp<sup>1</sup> and Arg<sup>579</sup> (the Arg<sup>579</sup>-Ala<sup>580</sup> bond is the endogenous cleavage site that gives rise to Vn<sub>57+65</sub>) (49). Exposure of the CKII-phosphorylated Vn to thrombin, which cleaves Vn specifically at the Arg<sup>506</sup>-Pro<sup>507</sup> bond (28) to yield a 57-kDa clipped Vn (Fig. 3A and B, lane 3), leaves the  $^{32}$ P label in the Vn<sub>57</sub> band, implying that the CKII phosphorylation site(s) in Vn, are located within the Asp<sup>1</sup> to Arg<sup>506</sup> segment of Vn. Finally, upon cleavage of CKII phosphorylated Vn at its acid-labile bond (Asp<sup>217</sup>-Pro<sup>218</sup>) leaves essentially all the label in the 40-kDa fragment (the large acid-cleavage-fragment of Vn containing the N terminus of intact Vn (49) (Fig. 3A, lane 4). In view of these results, we presumed that the CKII phosphorylation site(s) in Vn is located between residues Asp<sup>1</sup>-Arg<sup>217</sup> (Fig. 3B, lane 4).

**Sequencing Tryptic Peptides of CKII-phosphorylated Vn—**The commonly accepted consensus sequence for CKII phosphorylation is Ser/Thr-X-X-Glu/Asp (50). However, the presence of additional Asp or Glu residues at positions -3, +1, +2, +4, +5, or +7 (relative to the Ser or Thr residue to be phosphorylated) improves the phosphorylation efficacy ( $V_{max}/K_m$ ) (51, 52). In fact, most of the physiological substrates of CKII have at least one additional acidic amino acid residue in addition to the ones in the consensus sequence given above (50). Phosphoamino acid analysis of CKII-labeled Vn showed that this kinase phosphorylates Vn on Thr residues (Fig. 1B). There are four Thr residues in the 40-kDa fragment of Vn mentioned above that meet with the consensus sequence requirements of CKII targets: Thr<sup>57</sup>, Thr<sup>65</sup>, Thr<sup>57</sup>, and Thr<sup>65</sup>. Of these, Thr<sup>57</sup> and Thr<sup>65</sup> should be preferred targets for phosphorylation because they contain additional acidic residues in the preferred positions mentioned above.

In an attempt to identify the exact CKII phosphorylation site(s) in Vn, we digested the CKII-phosphorylated  $^{32}$ P-labeled Vn with trypsin, and the resulting mixture of tryptic peptides



**Fig. 2. Determination of the  $K_m$  and  $V_{max}$  values for the CKII phosphorylation of Vn in its native and in its heat-treated forms.** Native Vn and heat-treated Vn were phosphorylated by CKII in the presence of [ $\gamma$ - $^{32}$ P]ATP (see under "Materials and Methods") at the concentrations indicated in the Fig. (total reaction volume for each concentration, 50  $\mu$ l). After 2 min, the reaction was arrested by boiling for 3 min in Laemmli's sample buffer, and the products were subjected to SDS-PAGE (10% of acrylamide). Vn bands (both the Vn<sub>57</sub> and the Vn<sub>57+65</sub> forms together) were excised from the dried gel and counted in a scintillation counter. The  $V_{max}$  (A) and  $K_m$  values (B) for the CKII phosphorylation of native Vn (open circles) and heat-treated Vn (closed circles) were determined from the dependence of the initial velocity of the phosphorylation on the Vn concentration and a Lineweaver-Burk double reciprocal plot analysis.

was applied on an reverse phase-high pressure liquid chromatography C8 column, monitoring the absorbance of the peaks (at 214 nm) and their radioactivity. The emerging peak that had the highest radioactivity (57% of total) was analyzed and found to start with the sequence  $^{46}\text{GDFVFTMPEDPE}^{55}$  (data not shown), fitting the tryptic fragment  $^{46}\text{GDFVFTMPEDPE}^{55}\text{TVYD-DGEEK}^{55}$ . On the basis of this finding and the results summarized in Fig. 3, we concluded that the CKII phosphorylation sites in Vn are most likely Thr<sup>57</sup> or Thr<sup>65</sup>, or both. However, in view of the low yields and poor resolutions obtained when attempting to further degrade and purify the labeled peptides, we could not establish unequivocally which of these three possibilities is correct.

**Identifying the CKII Phosphorylation Site(s) in Vn by Single-site and Double-site Mutations—**In order to determine the site(s) of the CKII-phosphorylation in Vn, we expressed wt-Vn and other r-Vn s in a baculovirus expression system and obtained a set of Vn mutants in which each one of the two Thr residues mentioned above was replaced either by Ala or Glu. This resulted in the following r-Vn derivatives: T50A, T50E, T57A, and T57E (Fig. 4A), which we subjected to phosphorylation by CKII. As seen in Fig. 4, A and B, under the conditions of our experiment, the CKII phosphorylation was not completely abolished in any one of the four Vn mutants (T50A, T50E, T57A, and T57E). Therefore, we presumed that both threonines are probably targets for phosphorylation. To confirm this hypothesis, we constructed two double mutants in which both threonines were mutated either to Ala (T50A/T57A) or to Glu (T50E/T57E). Indeed, these two mutants were not phosphorylated by CKII (Fig. 4, A and B), demonstrating that

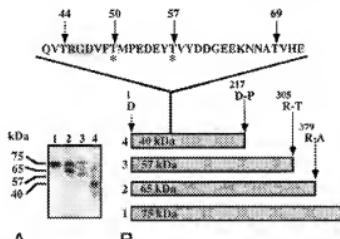


FIG. 3. Localization of the Thr residue(s) phosphorylated by CKII within the N terminus moiety of Vn. *A*, analysis of CKII phosphorylated Vn by SDS-PAGE. Native Vn (10  $\mu$ g) was phosphorylated *in vitro* either by PKA (100 ng) (lane 1) or by CKII (25 ng) (lanes 2–4) for 30 min at 22 °C in a reaction mixture (50  $\mu$ l) containing magnesium acetate (10 mM), [ $\gamma$ -<sup>32</sup>P]ATP (10  $\mu$ M, 6 Ci/mmol), and Hepes (50 mM), pH 7.5. *Lane 1*, intact Vn or isolated from plasma (only Vn<sub>40</sub> is seen because, under the conditions of our experiment, the Vn<sub>40</sub> was not phosphorylated by PKA in the absence of heparin). *Lane 2*, both the Vn<sub>40</sub> and the Vn<sub>40-20</sub> forms are seen in, indicating that the CKII phosphorylation site(s) is/are within Vn<sub>40</sub>. *Lane 3*, Vn was cleaved with thrombin (0.5 units), and which phosphorylated Vn<sub>40</sub> band is seen. *Lane 4*, acid-cleaved Vn, which yields Vn<sub>40</sub>. The reaction products obtained were subjected to SDS-PAGE (10% acrylamide), visualized by autoradiography, and analyzed. For experimental details, see also under "Materials and Methods." *B*, schematic presentation of the CKII phosphorylated Vns after proteolytic fragmentation. The products obtained after three different proteolytic degradations were analyzed. The scheme also illustrates the position of all of the Thr residues within Vn<sub>40</sub> that might be phosphorylated by CKII on the basis of its specificity.

these (and only these) threonines are the CKII phosphorylation sites in Vn. Under the conditions of the experiment depicted in Fig. 4, the incorporation of <sup>32</sup>P into wt-Vn was double, compared with each of the single mutations, T50A, T50E and T57A, which would be in line with the fact that the CKII phosphorylation occurs on both Thr<sup>50</sup> and Thr<sup>57</sup> (Fig. 4, *A* and *B*). However, the T57E mutant incorporated almost double the expected amount of <sup>32</sup>P into the remaining vacant site, Thr<sup>50</sup>. The reason for the enhanced incorporation of <sup>32</sup>P in the T57E mutant is apparently due to the fact that upon insertion of an acidic residue (a Glu residue at position +7 relative to Thr<sup>50</sup>), one obtains an improved CKII phosphorylation site in Thr<sup>50</sup>, as established in the systematic specificity studies of Pinna and co-workers (51, 52).

**The CKII Phosphorylation of Vn Enhances Its Ability to Promote Cell Adhesion and Spreading.**—To assess the effect of the CKII phosphorylation on Vn function, and thus on its possible physiological importance, we needed to compare a preparation of fully nonphosphorylated Vn with a preparation of fully phosphorylated Vn. For this comparison, we used nonphosphorylated wt-Vn with CKII-phosphorylated wt-Vn. In an attempt to achieve the highest stoichiometry in the phosphorylation of wt-Vn, we carried out the phosphorylation in the presence of poly-L-lysine, which has been shown to activate CKII *in vitro* (53). Indeed, as seen in Fig. 5 (*inset*), when poly-L-lysine was included in the phosphorylation reaction mixture, the incorporation of <sup>32</sup>P was enhanced, and it was targeted specifically to Thr<sup>50</sup> and Thr<sup>57</sup> sites, as indicated by the finding that no incorporation of <sup>32</sup>P occurred in the T50A/T57A mutant (Fig. 5, *inset*, *lane 4*). The stoichiometry of phosphorylation was calculated to be 1.7 mol of phosphate per mol of Vn. Because these two CKII phosphorylation sites in Vn are in the vicinity of its RGD sequence (positions 45–47), which is well known to be involved in cell adhesion and spreading (9, 10), we first tested the functional consequences of the CKII phosphorylation on

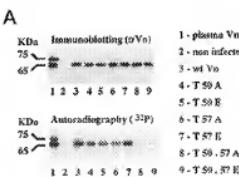


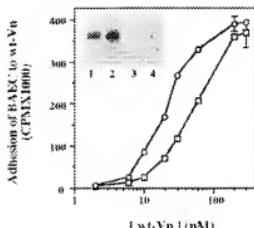
FIG. 4. Characterization of r-Vn and its mutants. *A*, characterization by immunoblotting and autoradiography of CKII phosphorylation. High-5 cells were infected with baculovirus containing wt-Vn or with the mutated Vns indicated in the figure. Samples of medium containing the secreted Vns were analyzed by immunoblotting using anti-human Vn polyclonal antibodies (*top panel*) and by CKII phosphorylation in the presence of 10  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (*bottom panel*). The reaction was arrested by boiling (5 min in Laemmli's sample buffer), and samples of the reaction mixtures were loaded on an SDS-polyacrylamide gel (10% acrylamide). The gels were dried and exposed to autoradiography (*bottom panel*). *B*, stoichiometry of the phosphorylation of r-Vn by CKII. Samples of Vns phosphorylated *in vitro* by CKII were loaded on an SDS-polyacrylamide gel (10% acrylamide). The gel was dried and exposed to autoradiography. The phosphorylated Vn bands were excised from the dried gel and counted on a scintillation counter. The calculation of the stoichiometry of phosphorylation was calculated on basis of the specific radioactivity of [ $\gamma$ -<sup>32</sup>P]ATP and the amount (in mol) of Vn applied in each lane.

cell adhesion. As shown in Fig. 5, the adhesion of BAECS to CKII-phosphorylated wt-Vn is significantly enhanced in comparison to the nonphosphorylated wt-Vn. It should be noted, however, that although the enhanced cell adhesion with CKII-phosphorylated wt-Vn was reproducible, its quantitation was erratic, presumably because the complete removal of poly-L-lysine was difficult in practice, and we found that poly-L-lysine interfered with the adhesion assays. We therefore sought further support to our finding using a molecular biology approach.

It was previously shown that mutation of serines or threonines in a protein to the negatively charged amino acid Glu yields a protein analog that often mimics the activity of that protein phosphorylated at these serines or threonines (54). The obvious advantages of such a mutant are that it constitutes a homogenous population of the phosphoprotein analog and that it can be used alongside a mutant in which the same residues are replaced by a nonphosphorylatable amino acid, such as alanine, which constitutes an appropriate control of the nonphosphorylated protein. To make use of the molecular biology approach in our case, we compared the double mutants T50E/T57E (representing the phosphorylated Vn) and the double mutant T50A/T57A (representing the fully nonphosphorylated Vn).

[<sup>35</sup>S]Methionine-labeled BAECS (or NIH-3T3 cells) were used to study the effect of introducing negatively charged amino acids (Glu residues) at positions 50 and 57 of Vn on its effectiveness in cell adhesion. The cells to be studied were placed on plates of either serially diluted wt-Vn or either one of

## CKII Phosphorylation of Vn Promotes Cell Adhesion

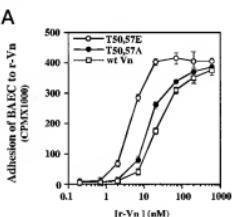


**Fig. 5.** Effect of CKII phosphorylated wt-Vn on BAEC adhesion compared with that of the nonphosphorylated wt-Vn. Adhesion of BAECs onto CKII phosphorylated wt-Vn and onto nonphosphorylated wt-Vn. Polystyrene plates were coated with increasing amounts of CKII-phosphorylated wt-Vn or phosphorylation in the presence of ATP (C) and with nonphosphorylated wt-Vn (which contained all of the components of the phosphorylation reaction mixture, except that ATP was substituted by AMP-PCP (D) (for details, see under "Materials and Methods"). [<sup>35</sup>S]Met-labeled BAECs were plated on top of the different substrata for 30 min. Cell adhesion was determined by counting the residual radioactivity after washing as described under "Materials and Methods." *Inset*, obtaining a high stoichiometry in the phosphorylation of wt-Vn. Samples of wt-Vn or Vn T50A/T57A were phosphorylated *in vitro* by CKII in the absence or presence of 15  $\mu$ g/ml poly-L-lysine (for details see under "Materials and Methods") and were loaded on an SDS-polyacrylamide gel (10% acrylamide). The gel was dried and exposed to autoradiography.

the mutants T50E/T57E or T50A/T57A for 30 min. In this experimental setup, the number of adhered cells was adequately monitored by the residual radioactivity remaining on the plates after washings, as described under "Materials and Methods." As seen in Fig. 6A, the adhesion of BAECs plated on T50E/T57E was several-fold higher than that of the mutant T50A/T57A (see, for example, the values at the r-Vn concentration of 7.5 nM). Similarly, the adhesion of NIH-3T3 cells to the T50E/T57E mutant was higher than that obtained with T50A/T57A (see, for example, values at the r-Vn concentration of 10 nM) (Fig. 6B). This difference in adhesion could not be attributed to a difference in the r-Vn bound to the plates, because the binding of these Vns to the polystyrene plates was found to be essentially identical (see Fig. 6C). It should be noted that some enhancement in adhesion was also observed with the T50A/T57A mutant (see Fig. 6, A and B), raising the possibility that the increased adhesion observed with T50E/T57E is not due exclusively to the charge inserted at the CKII phosphorylation sites. Therefore, we concluded that a comparison between T50E/T57E and T50A/T57A is probably more adequate than a comparison of T50E/T57E to wt-Vn.

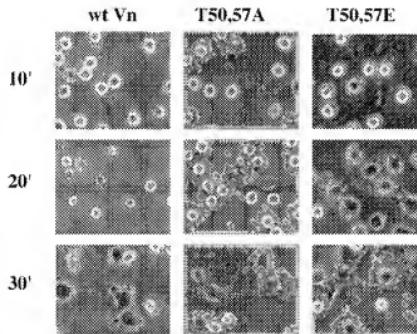
In view of the above evidence suggesting an involvement of the CKII phosphorylation of Vn in the control of its cell adhesion properties, we attempted to find out whether this phosphorylation might also modulate its involvement in cell spreading. Cells were plated on top of the different r-Vns (wt-Vn and the mutants mentioned above), and the spreading of cells was monitored under a phase microscope, with persistent warming (37 °C). The rate of spreading of both BAECs and NIH-3T3 cells on the T50E/T57E mutant of Vn was significantly faster than on the T50A/T57A mutant or on wt-Vn. As seen in Fig. 7, the NIH-3T3 cells that were plated on the various immobilized Vns became attached after 10 min (unattached cells were removed after 8 min by washing). However, after 20 min, only the cells plated on immobilized T50E/T57E were spread, suggesting that the CKII phosphorylation of Vn also enhances the rate of cell spreading.

*Evidence Suggesting an Involvement of  $\alpha_1\beta_3$  in the Enhanced*



**Fig. 6.** Effect of replacing Thr<sup>50</sup> and Thr<sup>57</sup> with Glu or Ala on the Vn-mediated cell adhesion of BAECs and of NIH-3T3 cells. Polystyrene plates were coated with increasing amounts of different r-Vns. [<sup>35</sup>S]Met-labeled BAECs or NIH-3T3 cells were plated on top of the different substrata for 30 min. Cell adhesion was determined by counting the residual radioactivity after washing as described under "Materials and Methods." *A*, adhesion of BAECs to wt-Vn, to its double mutant Vn T50E/T57E, and to its double mutant Vn T50A/T57A. *B*, adhesion of NIH-3T3 cells to wt-Vn, to its double mutant Vn T50E/T57E, and to its double mutant Vn T50A/T57A. *C*, determination of the r-Vn content on the polystyrene plates used in *panels A* and *B*. The comparison, carried out by enzyme-linked immunosorbent assay using anti-human Vn polyclonal antibodies, was made to secure that the differences observed in *panels A* and *B* are not due merely to differences in the adsorption efficacy of the various Vns onto the plastic plates.

*Adhesion of BAECs to Vn T50E/T57E*—Attempting to understand the mechanism through which the CKII-phosphorylation enhances the ability of Vn to promote cell adhesion and spreading, we considered two possibilities: (a) that the CKII phosphorylation modulates Vn function by enhancing the affinity between Vn and its specific integrin(s) on the cell in question; or (b) that upon phosphorylation, the binding of Vn is diverted, to another specific integrin. To find out which of these possibilities is correct, we used the integrin-specific antibodies (55), searching for the antibodies that would block the cell adhesion mediated by wt-Vn and by its two double mutants, Vn T50E/T57E and Vn T50A/T57A. The results presented in Fig. 8A indicate that the BAEC adhesion to wt-Vn, as well as to Vn



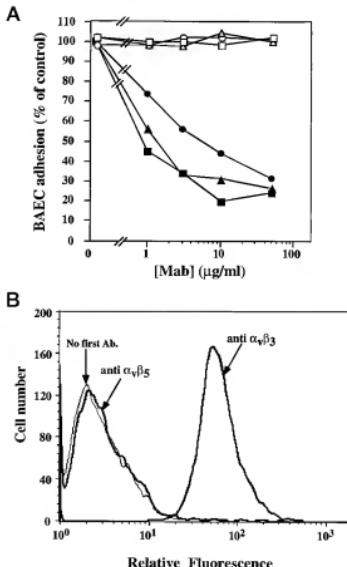
**Fig. 7.** Spreading of NIH-3T3 cells adhered to wt-Vn and to its double mutants Vn T50E/T57E and Vn T50A/T57A. Six-well Nunc plates were coated with the r-Vns indicated in the picture. Serum-starved NIH-3T3 cells were plated on top of wt-Vn and of its double mutants as described under "Materials and Methods." Cell spreading was monitored in a Zeiss phase microscope containing a heating unit plate calibrated at 37°C. Pictures were taken at different times (10, 20, or 30 min), as indicated in the figure.

T50E/T57E and Vn T50A/T57A, involves in these cells the  $\alpha_5\beta_3$  integrin, because in all three cases, the adhesion was found to be significantly inhibited by the anti- $\alpha_5\beta_3$  monoclonal antibodies. The fact that a higher concentration of antibodies was needed to achieve a similar inhibition of adhesion to Vn T50E/T57E can be attributed to a higher affinity of Vn T50E/T57E toward the  $\alpha_5\beta_3$  integrin. In line with this suggestion is the fact that the differences in adhesion are almost abolished when a higher concentration of anti- $\alpha_5\beta_3$  monoclonal antibodies was used (see the antibody concentration of 50  $\mu\text{g}/\text{ml}$  in Fig. 8). It should be emphasized that the lack of inhibition by the anti- $\alpha_5\beta_3$  cannot be taken as evidence to exclude the involvement of  $\alpha_5\beta_3$  in other cells, because the FACS analysis of the BAECS used here showed that they essentially do not express  $\alpha_5\beta_3$  (Fig. 8B).

#### DISCUSSION

Protein phosphorylation is well established as a key mechanism for intracellular regulation. However, in the last few years, several reports provided evidence to show that phosphorylation might also function as a regulatory device in the cell exterior (for a review, see Ref. 26). These reports included evidence that protein kinases are present outside the cell as ectoenzymes (on the cell surface) or exoenzymes (detached from the cell) and that they may well have outside the cell appropriate peptide or protein substrates (24, 25, 34–37). In addition, the occurrence of a release of ATP (a co-substrate of the kinases) into the cell exterior (56), the physiological circumstances under which the kinases and their substrates become exposed to each other, and, finally, the fact that phosphorylation modulates the functional properties of some of the kinase substrates support the notion that extracellular phosphorylation may well be a physiological regulatory mechanism. For example, we have shown that Vn is functionally modulated by PKA, which is released from platelets upon their physiological stimulation with thrombin (24–26), and similarly, that PKC phosphorylation of Vn attenuates its cleavage by plasmin (32).

Evidence for the existence of an extracellular CKII activity



**Fig. 8.** Identification of  $\alpha_5\beta_3$  as the integrin involved in the enhanced adhesion of BAECs. *A*, inhibition of BAEC adhesion to r-Vns by monoclonal antibodies against  $\alpha_5\beta_3$  but not against  $\alpha_6\beta_1$ . Polystyrene plates were coated with r-Vns (each at 5  $\mu\text{g}/\text{ml}$ ). [<sup>35</sup>Si]Met-labeled BAECs were preincubated (with shaking) for 30 min at 22°C, in the presence of increasing amounts of monoclonal antibodies against  $\alpha_5\beta_3$  (filled symbols) and against  $\alpha_6\beta_1$  (open symbols) before the adhesion assay was performed. The BAEC adhesion was determined by the reduced radioactivity on the plates after extensive washing, as described under "Materials and Methods." The percentage of adhered cells was calculated in comparison to a control of nonrelevant anti-hemagglutinin monoclonal antibodies. ■ and ▲, wt-Vn; ▲ and △, Vn T50A/T57A; ● and ○, Vn T50E/T57E. *B*, FACS analysis to determine the  $\alpha_5\beta_3$  and  $\alpha_6\beta_1$  integrins present in BAECs. BAECS were incubated with monoclonal antibodies directed against either  $\alpha_5\beta_3$  or  $\alpha_6\beta_1$ , followed by incubation with the secondary antibodies, fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Control BAECS were incubated with the secondary antibody only, as described under "Materials and Methods." The FACS analysis was performed with a Becton Dickinson FACScan, using the 530 filter. For each antibody, 5000 cells were analyzed.

was documented in several laboratories. Such kinase activity was found, for example, on epithelial cells (34, 35), on neutrophils (36, 37), on platelets (40, 41), and on endothelial cells (38, 39, 57). As in the case of PKA mentioned above, it was also shown that cells release a CKII-like activity in response to thrombin stimulation (39). Therefore, circulating blood proteins might well be physiological substrates for extracellular CKII. Indeed, the coagulation cofactors Va and VIII were shown to be substrates for the platelet-released CKII (40, 41), and the phosphorylation of fibrinogen by CKII was found to have an effect on the structure of the fibrin fibers formed after the fibrinogen cleavage by thrombin (58–60).

In this paper, we showed (i) that Vn is a substrate for CKII; (ii) that the CKII phosphorylation of Vn is selectively targeted to two Thr residues, Thr<sup>50</sup> and Thr<sup>57</sup>, which are vicinal to the integrin binding Arg-Gly-Asp sequence at positions 45–47 of

Vn; (iii) that the CKII phosphorylation of Vn is biochemically feasible *in vivo*, as it has a  $K_m$  of 0.5–2  $\mu\text{M}$ , lower than the Vn concentration in blood (3–6  $\mu\text{M}$ ); (iv) that the phosphorylation of Thr<sup>57</sup> facilitates the phosphorylation of Thr<sup>50</sup>, presumably by converting Thr<sup>50</sup> into a more preferred site for CKII; (v) that the stoichiometry of the CKII phosphorylation of plasma-Vn reaches a plateau at ~0.35 mol/mol, suggesting that *in vivo*, plasma-Vn is partially prephosphorylated (note, however, that at this stage it is not clear whether the CKII phosphorylation occurs intra- or extracellularly); (vi) that phosphorylation of Vn at positions Thr<sup>57</sup> and Thr<sup>50</sup> results in a significant modulation of one of its key physiological functions, cell adhesion and spreading, and (vii) that in BAECs, the enhancement of cell adhesion by the Vn T50E/T57E is mainly due to an increased affinity toward the  $\alpha_1\beta_1$  receptors.

The major implication of the findings reported here is that the CKII phosphorylation of Vn modulates a major physiological function of this protein, cell adhesion and spreading, and that this phosphorylation actually converts Vn from a cellular "glue" to a cellular "super glue." The exact mechanism involved in this modulation is not yet clear yet. However, we assume that the CKII phosphorylation of Vn (as in many other proteins) modulates the Vn structure in a way that favors the conformation(s) that exhibits the desired biological activity. In the case of Vn, this may be an "open" conformation or a "multimeric" form of this adhesive protein. It is commonly accepted that prior to the posttranslational modification, there is an equilibrium in which a certain (probably very low) percentage of the molecules exist in this active conformation. This percentage is usually responsible for the "basal" activity of an enzyme, for example. The role of the posttranslational modification is to establish a new equilibrium in which there is a greater percentage of molecules in the "active" conformation and consequently an increased rate of cell spreading. Further work is needed to establish that this modulation occurs *in vivo* as a physiological means of controlling the attachment and detachment of cells, and possibly in cell migration. We do hope that this report will set the stage for such study, which may also have important clinical implications.

Cell adhesion and spreading is now known to be involved in many diseases, including cancer (invasion and metastasis), thrombosis, inflammation, osteoporosis, proliferative retinopathy, etc. (61–65). In such diseases, cells often show alterations in their adhesive properties (66). Of special interest is the involvement of integrins in cancer (67). It was found that various tumor types stop producing specific integrins or display a different population of integrins, facilitating the migration of cells that normally do not migrate, providing them with means for invasion, and promoting the formation of new blood vessels (angiogenesis), thus giving the tumor access to the bloodstream and to nourishment (68–71). Molecular analysis of the specific integrins involved in these pathological conditions, and elucidating the regulatory mechanisms responsible for the control of cell adhesion to the substratum, or to each other, might shed light from a new angle on these diseases and be useful in the development of new drugs to challenge them.

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